

~~Exhibit~~ 2

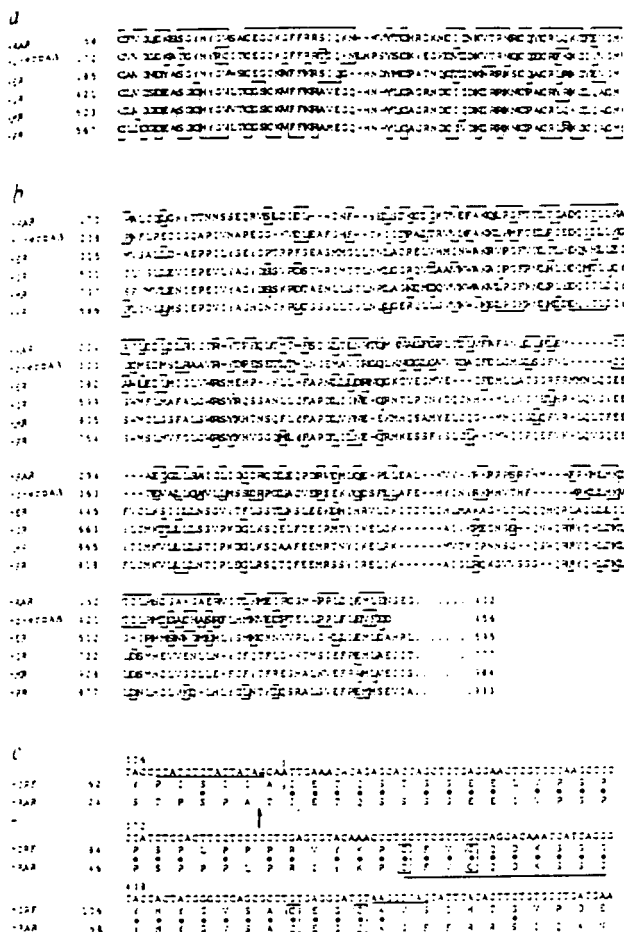


Fig. 3 Homology between the predicted sequences of hRAR and human nuclear receptors. *a*, The sequence of the putative DNA-binding domain (region C in Fig. 2) of hRAR is compared with those of the human nuclear receptors for thyroid hormone (hTbA)²⁷, oestrogen (hER)²⁵, glucocorticoid (hGR)²⁰, mineralocorticoid (hMR)²⁰ and progesterone (hPR)²¹. *b*, A similar alignment for region E of hRAR (Fig. 2) and the corresponding ligand-binding regions E of the same receptors. Dashes, spaces introduced into the sequences to obtain maximal alignment. Amino acid residues conserved between hRAR and at least one other receptor in these comparisons are boxed. *c*, Nucleotide and predicted amino acid sequences of the open reading frame for a novel human nuclear receptor-like genomic sequence (hORF), isolated from a hepatocellular carcinoma²⁴, aligned with the corresponding sequence of hRAR. The hORF nucleotide positions are numbered above the DNA sequence²⁴. Dots, amino acid residues conserved between the two sequences. Proposed acceptor and donor splice sites for the hORF DNA sequence are underlined at the top left and the bottom right of the alignment, respectively. The portion of hRAR sequence corresponding to region C is underlined. The conserved cysteine residues are boxed. Arrow, see text. Amino acid positions are numbered on the left-hand side.

the presence of glucocorticoids²². Thus we reasoned that replacing region C of hRAR by the corresponding region of hER might result in a RA-inducible *trans*-acting factor which could activate the transcription of a reporter gene normally responsive to oestrogen. The construction of this chimaeric receptor 'RAR-ER.CAS' is described in Fig. 5. HeLa cells transfected with RAR-ER.CAS show increased RA binding similar to that observed with RARO (data not shown). The putative *trans*-activating properties of the RAR-ER.CAS receptor were tested using the *vit-tk*-CAT receptor gene, which contains the oestrogen responsive element (ERE) from the *Xenopus* vitel-

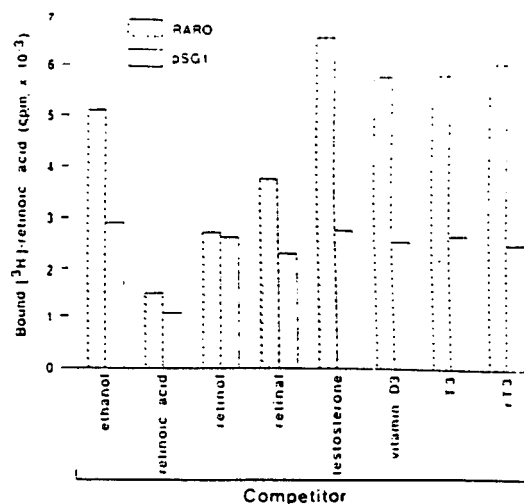


Fig. 4 Specific binding of RA to hRAR protein synthesized in HeLa cells transfected with the hRAR cDNA expression vector RARO. While cell lysates from HeLa cells transfected with either the *in vivo* expression vector RARO (see legend to Fig. 5) containing the hRAR cDNA open reading frame (hatched bars) or the parent vector pSG1 (open bars) were incubated with 17 nM [³H]RA in the presence of vehicle (ethanol) or a 230-fold excess of the unlabelled competitors as indicated. The amount of bound [³H]RA is shown.

Methods. HeLa cells (~10⁶ per dish) were transfected as previously described²⁵ with 20 µg of either the parent vector pSG1, an *in vivo* expression vector analogous to pKCR2²⁵ that contains the SV40 early promoter linked to a rabbit β-globin intervening sequence, or RARO. After 40 h, the cells were harvested, collected by centrifugation at low speed, washed with cold phosphate-buffered saline and resuspended in 120 µl (per dish) of 20 mM Tris-HCl, pH 7.2, 1 mM EDTA, 2 mM dithiothreitol (DTT), 50 mM NaCl and 0.3 mM phenylmethane sulphonyl fluoride (PMSF). The suspension was frozen and thawed once, and the cells were pelleted by centrifugation at 10,000 g for 15 min. All manipulations were carried out at 4°C. The supernatants from several dishes were pooled and the protein concentration was determined by Bradford assay. For the binding-competition assays, 1 µl of [³H]RA (New England Nuclear, 52.5 Ci mmol⁻¹) was added to 50 µl of lysate, giving a final concentration of 17 nM. Competing ligand (2 µl) was added to a final concentration of 4.2 µM. The competitors tested were all *trans*-retinoic acid, all *trans*-retinol, all *trans*-retinal, testosterone, L-3,5,3'-triiodothyronine (T3), L-3,3',5'-triiodothyronine (rT3) (Sigma) or 1,25-dihydroxyvitamin D3 (Hoffman-La Roche). The lysates were incubated for 3 h at 4°C and mixed with 100 µl of a cold dextran-coated charcoal suspension for 15 min. Unbound [³H]RA was removed by centrifugation at 10,000 g for 3 min at 4°C and 100 µl of the supernatant was taken for scintillation counting. Values shown are from duplicate experiments and were corrected for differences in protein concentration between the pSG1-transfected and RARO-transfected pooled lysates.

logen A2 gene (*vit*) located upstream of the herpes virus thymidine kinase promoter (*tk*) and the *Escherichia coli* chloramphenicol acetyl transferase (CAT) gene²¹.

Cotransfection of HeLa cells with the chimaeric receptor RAR-ER.CAS and the *vit-tk*-CAT reporter gene resulted in an increase of CAT activity which was dependent on the presence of 10⁻⁷ M RA, but not of oestradiol (Fig. 6*a*, lanes 7 and 8, 9-11, and 12 and 13; three different transfection experiments). Using 1 µg of *vit-tk*-CAT construct, maximal stimulation was achieved with ~100 ng of RAR-ER.CAS (Fig. 6*b*). We note that the stimulation of *vit-tk*-CAT expression by the RAR-ER.CAS chimaeric receptor in the presence of RA is comparable to that brought about under similar conditions by the hER expression vector HEO in the presence of oestradiol (Fig. 6*a*, lanes 12-15; HeLa cells transfected under these conditions contained on